Peptide mapping is a critical step in the characterization of therapeutic proteins for post-translational modifications. The use of automated high-throughput workflows has significantly improved the efficiency and reproducibility of this process. In the study by Millán-Martín et al. (2018), they introduce a method that achieves 100% sequence coverage in under 15 minutes using the Thermo Scientific™ KingFisher™ Duo Prime Purification System. This system simplifies the digestion process and ensures reliable and reproducible peptide maps, which is crucial for the development of high-quality biopharmaceuticals.

**ABSTRACT**

Peptide mapping is used to identify and monitor several critical quality attributes (CQAs) and is thus indispensable for the characterization and comparison of biopharmaceuticals. The analysis is used to confirm that the correct sequence has been expressed for the protein and to check for post-translational and chemical modifications that may be introduced during the manufacturing and storage of the product. In-depth characterization techniques offer mass spectrometry (MS) coupled to liquid chromatography for the peptide analysis. However, many QC methods rely exclusively on detection by ultraviolet (UV) absorption after the peptides have been identified by MS.

Proteolytic digestion is a shared bottleneck during sample preparation for both targeted bioanalysis and peptide mapping analysis. Established methods often rely on time and labor due to many steps involved in the protocol and may negatively affect the reproducibility and reliability of the entire chromatography workflow.

This work details on an automated digestion workflow that mitigates against manual errors during sample preparation. The Thermo Scientific™ SMART Digest™ heat-stable trypsin allows for a fast digestion and has been shown to facilitate reliable and reproducible peptide maps and increased sequence coverage in targeted mass spectrometry analyses [1]. The optimal low pH SMART Digest buffer allows for the simultaneous reduction and heat-denaturation in one step with the peptides at 70°C. The digestion, induced modifications are excluded due to its optimized buffer formulation. Automation of the digestion is achieved by using a magnetic bead support to immobilize the SMART Digest trypsin that allows its use in combination with the Thermo Scientific™ KingFisher Duo Prime magnetic bead purification system. The automated workflow is demonstrated to produce high-resolution peptide maps with superior performance and reproducibility.

**INTRODUCTION**

Peptide mapping is becoming more important in the industry with the increasing popularity of multi-attribute monitoring techniques. This poster describes a reliable and well-characterized method that is applicable for mass spectrometry and is suitable for routine analysis.

Thermotrapped trypsin is the enzyme most commonly used for proteolytic digestion due to its high specificity. Although a widely accepted technique, in-solution trypsin digestion protocols require for sample preparation labor intensive and prone to errors. These errors affect the quality of the analytical data compromising the ability to reproduce, characterize a protein to the required standard. Automation of the sample preparation with low sample preparation induced modifications has never been that successful. The digestion must be reproducible and the analysis extremely stable to allow unambiguous characterization through peptide mapping. The workflow has to overcome these limitations and define the automated protein digestion of mAb drug products. The standardization and reproducibility of the method is a prerequisite for the fingerprinting and identification of mAbs and is improved by automation. The Thermo Scientific™ SMART Digest™ kit and the Thermo Scientific™ KingFisher Duo Prime purification system was used to automate the digestion process. Digestion, Reduction and Glycation were investigated in detail. Sample preparation techniques are discussed to create understandable modifications that may result in an overestimation of the quantified attribute. Attention was therefore made to ensure a low level of sample-induced modifications. An optimized buffer formulation is used to allow semi-automated and automatic digestion even at a low pH to achieve the constant reduction of disulfide bridges with TCEP during digestion, while suppressing digestion-induced degradation even further.

**MATERIALS AND METHODS**

**Equipment and Consumables**

Thermo Scientific™ KingFisher™ Duo Prime Purification System, Thermo Scientific™ Vanquish™ Flex or Horizon UHPLC System, Thermo Scientific™ Q Exactive™ Plus with BioPharma option, Thermo Scientific™ Accela™ VANQUISH™ C18 (2.2 µm, column, 21 x 250 mm) Thermo Scientific™ SMART Digest™ Trypsin standard or low pH Kit, Magnetic Bead option

**Data Analysis**

Thermo Scientific™ BioPharma Finder 3.0 software, Thermo Scientific™ Chromelon CDS 7.2, Thermo Scientific™ Xcalibur™ v 2.2

**Sample Preparation**

Commercially available monoclonal antibody samples Abalumab and MAb were dialyzed as supplied to 2 mg/ml for digestion. Automated magnetic SMART Digest, Manual SMART Digest, In-solution digest and Rapid Digestion conditions are described in ref 1.

**RESULTS**

Using the magnetic SMART Digest kit in combination with the KingFisher Duo Prime system simplifies the digestion process and reduces the time from trypsin peptide mapping sample preparation compared to established alternative protocols. Complete digestion can be achieved in under 45 min (Fig 2a), resulting outstanding reproducibility and 100% sequence coverage for all mAb products tested (Fig 2b), demonstrating the robustness and reliability of the automated approach. Standard relative retention reproducibility is ≤ 5% RSD with an average of 0.35% is achieved when using the Vanquish Flex Linear UHPLC system for the technical digestion replicates [2] and RSD values for the relative peak area was found to be less than 5% for the majority of peptides [3]. While the extent of tryptic autolysis is very limited by the immobilization of the enzyme, a time-dependency is nevertheless observable, with the optimal digest time for mAb usually being in the range of 45 min. The automated digestion approach using the KingFisher Duo Prime system easily allows to implement a time-course method that consistently facilitates a time-course study for digestion time optimization [4].

**CONCLUSIONS**

- **The Magnetic SMART Digest kit provides simple, automated, rapid protein digestion for peptide mapping analysis and FTMS quantification for in-depth biopharmaceutical characterization and comparability studies between innovator and biosimilar products.**
- **Peptide mapping was easily automated, resulting in less sample handling, increased productivity, and improved reproducibility, even with peptides at low levels. This will allow confident transfer of methods between laboratories.**
- **The new low pH SMART Digest Kit further minimizes the amount of digestion-induced deamidation at 70°C.**
- **Supplementing the new low pH SMART Digest buffer with TCEP allows for concomitant reduction and heat-denaturation in one step parallel to the digestion.**
- **The very low levels of method-induced modifications observed for the automated SMART Digest method make the approach suitable for its application in multi attribute monitoring techniques involving peptide mapping analysis.**

**REFERENCES**


**TRADEMARKS/LICENSES**

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